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(54) Title: GENE-TARGETED NON-HUMAN MAMMALS DEFICIENT IN THE SOD-1 GENE

(57) Abstract

Gene-targeted heterozygous and homozygous SOD-1 null non-human mammals, methods for producing them, and methods for use are described. Deletion vectors and gene-targeted cells are also described, as are methods for producing and using the same.

GENE-TARGETED NON-HUMAN MAMMALS DEFICIENT IN THE SOD-1 GENE

Field of the Invention

This invention generally concerns gene-targeted non-human mammals; particularly, gene-targeted mice
5 deficient in the normal SOD-1 gene; and, more particularly, gene-targeted mice deficient in the normal SOD-1 gene incapable of expressing the Cu/Zn SOD protein.

Background of the Invention

The molecular reduction of oxygen to water during
10 oxidative phosphorylation results inevitably in the production of superoxide radicals ("$O_2^{\bullet-}$") that are reactive oxygen species containing an unpaired electron orbital. Superoxides act as either reductants or oxidants and can form other reactive species including the hydroxyl radical
15 ("$OH^{\bullet}$") through interaction with iron (Haber-Weiss reaction) and peroxynitrite by reaction with nitric oxide. Reactive oxygen species attack proteins, DNA, and membrane lipids, thereby disrupting cellular function and integrity.

The primary defenses against the superoxide
20 radicals are the superoxide dismutase enzymes (SOD) that catalyze the dismutation of superoxide to hydrogen peroxide. Three forms of SOD are known to exist in mammals: cytoplasmic SOD (Cu/Zn SOD), mitochondrial SOD (Mn SOD), and extracellular Cu/Zn SOD (EC-SOD). In mammals, SOD-1 refers
25 to the gene that encodes Cu/Zn SOD, SOD-2 refers to the gene that encodes Mn SOD, and SOD-3 refers to the gene that encodes EC-SOD.

Cu/Zn SOD is a homodimeric protein of 32 kD that is localized to the cytoplasm and, perhaps, peroxisomes. It is

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produced constitutively in all cell types and is the most abundant SOD. High to moderate levels of Cu/Zn SOD are found in erythrocytes, the liver, skeletal muscle, and the brain. Mn SOD is a tetrameric protein localized to

5   mitochondria and is found at approximately 5 to 10% of the levels of Cu/Zn SOD in cells. EC-SOD is a tetrameric protein evolutionarily related to Cu/Zn SOD that is found at low levels in plasma.

SOD-1 has been isolated and cloned from many

10 different organisms. The complete amino acid sequences of Cu/Zn SOD from 11 different species have been compared. A high degree of homology is evident among those of vertebrate origin and the metal binding sites appear to be conserved in all the species. (Bannister et al., *CRC Critical Reviews in*

15 *Biochemistry*, 22(2): 111-180, 1987, incorporated herein by reference.)

The human Cu/Zn SOD has 153 amino acids per monomeric subunit and is encoded by a single-copy gene on chromosome 21. (Tan et al., *J. Exp. Med.*, 137: 317-330,

20 1973.) Human SOD-1 and cDNA have been cloned and sequenced. (U.S. Patent No. 5,196,335, issued to Yoram Groner on March 23, 1993; U.S. Patent No. 5,252,476, issued to Hallewell et al. on October 12, 1993, both incorporated herein by reference.) A full-length cDNA for murine Cu/Zn SOD has

25 been isolated (Bewley, G. C., *Nucleic Acids Res.*, 16:2728, 1988, incorporated herein by reference) and the structure of the single-copy gene on chromosome 16 has been reported. (Benedetto et al., *Gene*, 99: 191-195, 1991.)

Oxidative stress has been implicated in normal

30 aging and many human pathological conditions. (J.M.C. Gutteridge, *Free Rad. Res. Comms.*, 19: 141-158, 1993, incorporated herein by reference; Halliwell and Gutteridge, Methods in Enzymology, Vol. 186, pp. 1-75, 1990.) Some examples include stroke, head and spinal cord trauma,

35 Alzheimer's disease, atherosclerosis, Parkinson's disease, and Huntington's disease. Major sources of free radical production, including ischemia/reperfusion, inflammation,

and mitochondrial injury, are common features of many of
these conditions. Diseases in which reduced SOD activity
may play a role include, for example, amyotrophic lateral
sclerosis (ALS), Parkinson's disease, Fanconi's anemia and
5   aluminum toxicity.

         A detrimental role for the superoxide radical in
human disease is supported in animal models of disease
processes using transgenic mice overexpressing Cu/Zn SOD.
Chan et al., *Acta Neurochirurgica. Suppl.*, 51: 245-247,
10   1990, reported that cortical neurons isolated from
transgenic mice overexpressing Cu/Zn SOD two to threefold
relative to normal Cu/Zn SOD levels are protected against
glutamate neurotoxicity *in vitro.* Neuroprotection is also
conferred in Cu/Zn SOD transgenic mice against focal
15   cerebral ischemia (Kinouchi et al., *Proc. Natl. Acad. Sci.
USA*, 88: 11158-11162, 1991) and N-methyl-4-phenyl-1,2,3,6-
tetrahydropyridine (MPTP) induced toxicity that causes
damage similar to that observed in Parkinson's disease
(Przedborski et al., *J. Neurosci.*, 12: 1658-1667, 1992).
20   Evidence for a direct role of Cu/Zn SOD in human
disease is exemplified by the disease ALS. ALS is a
progressive paralytic disorder caused by the degeneration of
large motor neurons of the brain and spinal cord and is
usually fatal within five years of onset of symptoms.
25   Approximately 90% of ALS is "sporadic", i.e., no familial
history of the disease. Enhanced oxidative damage and
stress in sporadic ALS patients, as evidenced by increases
in protein carbonyl content and complex I electron transport
activity, was reported by Bowling et al., *J. Neurochem.*,
30   61(6): 2322-2325, 1993. Approximately 10 % of ALS is
inherited as an autosomal dominant trait and is termed
familial ALS (FALS). Recently, in a subset of FALS cases,
16 different missense mutations were identified within SOD-1
that resulted in a 40 to 50% reduction in the Cu/Zn SOD
35   activity measured in red blood cell lysates. (Rosen et al.,
Nature, 362: 59-62, 1993; Deng, H.X. et al., *Science* 261:
1047-1051, 1993.)

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The role of reduced Cu/Zn SOD activity in FALS is
unclear, however, because transgenic mice overexpressing
human Cu/Zn SOD bearing one of the FALS mutations develop
progressive motor neuron loss similar to that observed in
5   the human condition. (Gurney et al., *Science*, 264: 1772-
1775, 1994). Ripps et al., *Proc. Natl. Acad. Sci. USA*, 92:
689-693, 1995, report that transgenic mice bearing a
mutation in the mouse SOD-1 gene that corresponds to one of
the changes in human FALS gene have high expression of the
10   altered gene in the central nervous system which is
associated with an age-related rapidly progressive decline
of motor function accompanied by degenerative changes of
motorneurons within the spinal cord, brain stem, and
neocortex. The tissues of these transgenic mice had normal
15   levels of total SOD activity. This suggests that the
mutations confer a gain-of-function on the Cu/Zn SOD protein
that contributes to disease onset. One possibility is that
reduced Cu/Zn SOD activity measured in the FALS patients is
a co-factor in the disease. (Gurney, *Science*, 266:1587,
20   1994.)

To determine whether decreased SOD activity could
contribute to motor neuron loss, Cu/Zn SOD was inhibited
chronically with antisense oligonucleotides or
diethyldithiocarbamate in spinal cord organotypic cultures
25   derived from rats. Chronic inhibition of Cu/Zn SOD resulted
in the apoptotic degeneration of spinal neurons, including
motor neurons. Motor neuron toxicity could be entirely
prevented by the antioxidant N-acetylcysteine. (Rothstein
et al., *Proc. Natl. Acad. Sci. USA*, 91: 4155-4159, 1994.)
30   Similarly, Troy et al., *Proc. Natl. Acad. Sci. USA*, 91:
6384-6387, 1994, reported that inhibition of Cu/Zn SOD
synthesis by antisense oligonucleotides in cultured PC12
cells (rat pheochromocytoma cells) results in apoptotic-like
cell death in undifferentiated and nerve growth factor(NGF)-
35   differentiated cultures. The authors suggest that free
radical production caused by inhibition of Cu/Zn SOD is
responsible for induction of the cell death pathway.

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An animal model deficient in, or completely
lacking, Cu/Zn SOD, would facilitate the elucidation of the
role of Cu/Zn SOD and superoxide radicals in disease, and
the testing of counteractive compounds. The development of
5 an animal model affords both *in vivo* and *in vitro* testing
opportunities as cultured cells from the SOD-1 deficient or
lacking animals can be utilized for the *in vitro* testing.
An animal model deficient in Cu/Zn SOD will permit one to
determine whether there are compensatory responses of other
10 anti-oxidant systems, including Mn SOD and EC-SOD.

It has been proposed that SOD is essential for
normal aerobic life. (C. W. Olanow, *TINS*, 16: 439-444,
1993.) For example, non-mammalian SOD deficient organisms
have been established which exhibit highly deleterious
15 characteristics. *Escherichia coli* lacking SOD activity
exhibit an oxygen-dependent auxotrophy for branched chain
amino acids. These organisms are unable to grow aerobically
on minimal media, and are highly sensitive to the free
radical-producing agents paraquat and hydrogen peroxide.
20 (Carlioz et al., *EMBO J.*, 5: 623-630, 1986.) Cu/Zn SOD
deficient yeast (*Saccharomyces cerevisiae*) are intolerant to
atmospheric levels of oxygen and are auxotrophic for lysine
and methionine. (Chang et al., *JBC*, 266: 4417-4424, 1991.)
Null mutations for Cu/Zn SOD in *Drosophila melanogaster*
25 cause toxic hypersensitivities to oxidative stress
conditions and a significant reduction in the adult
lifespan. (Phillips et al., *Proc. Natl. Acad. Sci. USA*, 86:
2761-2765, 1989.)

30 **Summary of the Invention**
In one aspect, the present invention relates to
heterozygous SOD-1 null non-human mammals, preferably
exemplified by a gene-targeted mouse lacking one normal copy
(allele) of SOD-1, thereby producing a reduced amount of
35 Cu/Zn SOD. In a preferred aspect, the invention relates to
homozygous SOD-1 null non-human mammals exemplified by a
gene-targeted mouse lacking both normal copies (alleles) of

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SOD-1, thereby producing no measurable amount of Cu/Zn SOD
protein.

In another aspect, the present invention relates to
a recombinant DNA construct for effecting the gene targeting
by means of homologous recombination.

In yet another aspect, the present invention
relates to methods for testing the effectiveness of
compounds in counteracting the effects of oxidative stress.

In a further aspect, the present invention relates
to gene-targeted cells lacking at least one normal SOD-1
allele.

Brief description of the drawings

Figure 1 depicts a prototype gene targeting
strategy.

Figure 2 depicts mouse SOD-1 genomic clone maps
(2a) and a composite determined therefrom (2b).

Figure 3 depicts restriction mapping with the
FLASH® Nonradioactive Gene Mapping Kit. A typical
restriction map for a genomic clone isolated from a Lambda
DASH® II library is shown at the top of the figure.

Figure 4 depicts the SOD genomic map (4a) and
targeting vector(4b).

Figure 5 depicts the construction of intermediate
plasmid pPNTlox^2.

Figure 6 depicts the construction of intermediate
plasmid pSK18-9.

Figure 7 depicts the construction of intermediate
plasmid pSOD3'homolTV.

Figure 8 depicts the construction of intermediate
plasmid pSK EH69-2.

Figure 9 depicts the construction of deletion
vector pSOD-TV.

Figure 10 depicts the strategy used to detect
homologous recombination within mouse SOD-1.

Figure 11 depicts the measurement of Cu/Zn SOD
concentration and activity levels in blood samples from

wild-type mice, and mice heterozygous and homozygous for the
SOD-1 null allele.

Detailed Description

Because SOD is the initial defense against oxygen
5   toxicity, and cytoplasmic Cu/Zn SOD represents a large
fraction of SOD activity in mammals, it was not predictable
whether mammals completely lacking Cu/Zn SOD, i.e., null for
both alleles, could survive. With the advent of methods for
introducing gene-targeted mutations in mammals currently
10 exemplified in the art using mice, it was of interest to
determine whether ablation or "knock-out" of the mouse
normal SOD-1 gene could be accomplished resulting in gene-
targeted, non-human mammals deficient in or lacking
cytoplasmic Cu/Zn SOD activity, and whether mammals lacking
15 Cu/Zn SOD activity would be able to survive. Such
mutagenized mammals are useful for directly addressing the
role of oxidative stress in any number of pathological
conditions -- i.e., aging, radiation damage, immunological
dysfunction, and neurological disorders -- in which free
20 radical damage has been implicated.

As disclosed in more detail below, gene-targeted
mammals according to the invention lacking one normal copy
of SOD-1 (or SOD-1 allele) produce a reduced amount of Cu/Zn
SOD. Such mammals are defined herein as "deficient" in SOD-
25 1, and are termed "heterozygous SOD-1 null." Mammals
according to the invention lacking both copies of normal
SOD-1 produce substantially no, or no measurable amount, of
Cu/Zn SOD. Such mammals are defined herein as "lacking"
SOD-1, and are termed "homozygous SOD-1 null."
30   As used herein, the phrase "normal" or "normal
copy" in reference to SOD-1 or SOD-1 allele means the gene
encoding wild type amounts of enzymatically active Cu/Zn SOD
protein in a wild type mammal whose genome includes such
SOD-1. Thus, an animal lacking at least one normal copy of
35 an allele, as defined herein, need not necessarily have that
allele excised form the genome of that animal; rather, the

gene sequence can be sufficiently disrupted such that the expression of a protein encoded thereby is disrupted. Therefore, a mammal lacking at least one copy of a normal SOD-1 allele can, as defined herein, have a mutated SOD-1

5 allele that disrupts expression of the Cu/Zn SOD.

As used herein, the term "reduced amount" in reference to the amount of Cu/Zn SOD protein expressed in a gene-targeted mammal lacking one normal copy of SOD-1 gene means between about 25% and about 75% of wild-type Cu/Zn SOD

10 protein typically expressed in a comparative mammal (e.g., a mouse in the case of the gene-targeted mouse).

As used herein, the term "no measurable amount" in reference to the amount of Cu/Zn SOD protein expressed in a gene-targeted mammal lacking both copies of normal SOD-1

15 means less than about 10% of wild-type Cu/ZN SOD protein normally expressed in a comparative mammal.

Methodologies for measurement of protein expressed by a gene are varied and well-known; analyses may be made, for example, using anti-Cu/Zn SOD protein antibody

20 measurements of tissue samples such as exemplified in Example 5 below.

As used herein, the term "about" in reference to a numerical value means "+/- 10%" of the numerical value, e.g., "about 10%" means between 9% and 11%.

25 As used herein, the term "control" is given the definition ordinarily ascribed to the word in a scientific setting; thus, in situations where a putative therapeutic compound is provided to, e.g., a homozygous SOD-1 null mouse, to determine the effectiveness of the compound for

30 counteracting the deleterious effects of oxidative stress, a "control" for such an analysis could be, e.g., a homozygous SOD-1 null mouse that is not provided with the compound; a SOD-1 null mammalian cell that is not provided with the compound, etc.

35 To implicate oxidative stress as an etiological factor in human neurological disease, deficits that develop

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spontaneously in specific neuronal populations vulnerable to
disease can be evaluated during normal aging. Examples of
some deficits include the accumulation of oxidative damage
on protein and enzymes (Reznick et al., Methods in
5   Enzymolology, Vol. 233, p. 363, 1994; Bowling et al.,
supra), DNA (Sanchez-Ramos et al., Neurodegen., 3: 197-204,
1994) and membrane lipids (Dousset et al., Methods in
Enzymolology, Vol. 244, pp.459-469, 1994). Additional
deficits include neuronal cell loss and behavioral
10 abnormalities (Walsh and Cummins, Physcol. Bull., 83: 482-
504, 1976). More specifically, the influence of oxidative
stress on apoptotic cell death in vivo can be examined in
the SOD-1 deficient and SOD-1 lacking mammals during normal
aging or after pharmacological lesions of particular
15 neuronal populations. Apoptosis is established by measuring
DNA cleavage, nuclear condensation, and cellular morphology
(Rothstein et al., Proc. Natl. Acad. Sci. USA, 91: 4155-
4159, 1994). Pharmacological lesions of interest include
excitotoxicity (Miyamoto et al., Exp. Neurol., 108: 38-45,
20 1990; Bazzett et al., Exp. Neurol. 120: 177-185, 1993),
focal ischemia (Kinouchi et al., Proc. Natl. Acad. Sci. USA,
88: 11158-11162, 1991) and facial motor neuron axotomy (Yan
et al., J. Neurobiol., 24: 1555-1577, 1993).
            Having established viable gene-targeted mammals
25 which are both heterozygous SOD-1 null and homozygous SOD-1
null, analysis of the effects of having a reduced amount of
Cu/Zn SOD protein, and no measurable amount of Cu/Zn SOD
protein, respectively, on the development and behavior of
such mammals can be determined. Oxidative stress can be
30 potentiated by providing the heterozygotes and homozygotes a
vitamin E/selenium deficient diet, exercise, and/or exposure
to hyperbaric oxygen. Putative therapeutic agents for
countering the effects of a Cu/Zn SOD deficiency can be
analyzed and screened. Furthermore, the mammals can be used
35 as a source of cells for culture for in vitro testing.
Those skilled in the art will readily recognize the myriad
of available choices for positive utilization of such

mammals.

Administration of the compound to be tested may be carried out by any appropriate means. In the case of the gene-targeted mammals, for example, administration may be
5 intracerebrally and/or intracerebroventricularly, and/or intravascularly, intralymphatically, parenterally, subcutaneously, intramuscularly, intraperitoneally, intrastitially, hyperbarically, and the like, using a variety of dosage forms and amounts, the particular route of
10 administration and the dosage form and amount being dependent on various factors such as the type of compound being tested, the age, weight, and type of mammal model etc. In the case of the gene-targeted cells, administration of the compound to be tested may be carried out, if desired, by
15 simply placing the compound in the cell culture medium. Generally, a lower dosage amount will be employed with the gene-targeted cells than with the gene-targeted mammals.

Deletion of the SOD-1 gene in a mouse genome is specifically disclosed below. Currently, gene-targeting
20 protocols utilized in the art are defined by the mouse; however, as the state of the gene-targeting art progresses to other mammals (i.e., rats, pigs, rabbits, non-human primates), the technique and methods disclosed below can rapidly be adapted thereto.

25 The deletion of SOD-1 was accomplished by first effecting the deletion of SOD-1 in embryonic stem (ES) cells using homologous recombination, resulting in mutagenized ES cells. The SOD-1 deletion was then incorporated into a mouse by introducing the mutagenized ES cells into a
30 developing mouse embryo. Breeding the resulting chimeric mice and their progeny produced some mice which were lacking one normal copy of SOD-1 (heterozygotes) and others which were lacking both normal copies (homozygotes).

Although we disclose herein our most preferred
35 strategy for the development of heterozygous SOD-1 null non-human mammals and homozygous SOD-1 null non-human mammals using a most preferred gene-targeting vector ("deletion

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vector"), other strategies and targeting vectors will be
readily apparent to those skilled in the art. Thus, the
following disclosure is neither intended to be, nor to be
construed as, a limitation on the disclosure or the claims
5 to follow.

Figure 1 depicts a general paradigm for introducing
deletions into a mammalian genome using homologous
recombination, as has been reviewed by Capecchi, M. R.,
Trends Genet, 5: 70-76, 1989 and Koller and Smithies, *Ann.*
10 *Rev. Immunol.*, 10: 705-730, 1992, both incorporated herein
by reference. However, other methods can be utilized for
mutagenizing the mammalian genome. These include other
methods of gene-targeting (for examples, see Capecchi,
supra; Koeller and Smithies, *supra*) chemical mutagenesis
15 (Rinchik, *Trends Genet.*, 7: 15-21, 1991) and insertional
inactivation of genes by the random integration of vectors
such as retroviruses (Robertson et al., Ciba Found Symp.,
165: 237-250, 1992).

A length of genomic DNA is first depicted by
20 organizing it into regions (numbered 0 - 6 in Figure 1a).
In Figure 1, region 3 is designated to be deleted.
Homologous recombination using a gene-targeting vector is
utilized. The type of gene-targeting vector used for the
deletion of a gene is termed a replacement or deletion
25 vector.

"Deletion vector" as used herein refers to a vector
that includes one or more selectable marker sequences and
two sequences of DNA homologous to the genomic DNA that
flank the DNA gene sequence which is to be deleted. An
30 "homologous sequence" as used herein is defined as a
sequence at least about 90%, but preferably about 95%,
identical to the corresponding target sequence. These
flanking sequences are termed "arms of homology." In Figure
1b, the arms of homology are represented by regions 1-2 and
35 4-5. Preferably, these arms of homology are substantially
isogenic for the corresponding flanking sequences in the
cell being targeted or "target cell." A "substantially

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isogenic" sequence is at least about 97-98% identical to the
corresponding target sequence. The use of DNA isogenic to
the target cells helps assure high efficiency of
recombination with the target sequences. (teRiele et al.,
5 *Proc. Natl. Acad. Sci. USA*, 89: 5128-5132, 1992.) The
cumulative region of homology is longer than about 50 bp but
is preferably about 2 kb or greater.

        The deletion vector preferably includes at least a
positive selection marker within the arms of homology to
10 enable the scoring of recombination. Such positive
selection markers can confer a phenotype not normally
exhibited by wild-type mammals; for example, resistance to a
substance normally toxic to the target cell. In this
example, the positive selection marker can be flanked by
15 loxP sites allowing for excision of the positive selection
marker; see, for example, U.S. patent No. 4,959,317, issued
to B. L. Sauer on September 29, 1990, incorporated herein by
reference. In a further preferred embodiment, the deletion
vector also includes one or more negative selection markers
20 outside the arms of homology to facilitate identification of
proper homologous recombinants. The negative selection
markers can confer, for example, sensitivity to a substance
not normally toxic to the target cell. The selection
markers can be gene cassettes --ie., include both a promoter
25 and an accompanying coding sequence. The result of
homologous recombination of the gene-targeting vector with
cellular DNA in the paradigm is shown in Figure 1c. As
depicted therein, region 3 has been replaced by the positive
selection marker.

30   In the specific deletion vector according to the
present invention, the positive selection marker is neo^r, a
gene that encodes resistance to the neomycin analog G418,
and the negative selection marker is the herpes simplex
virus thymidine kinase gene (HSV-TK), a gene that encodes
35 susceptibility to ganciclovir. Upon successful gene-
targeting and homologous recombination, the positive
selection marker is incorporated into the genome in place of

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the gene to be deleted within the arms of homology, thereby
making the gene-targeted cells resistant to G418, while the
negative selection marker is excluded, thereby maintaining
the cells' resistance to ganciclovir. Thus, to enrich for
5   homologous recombinants, gene-targeted cells are grown in
culture medium containing G418 to select for the presence of
the neo^r gene, and ganciclovir to select for the absence of
the HSV-TK gene.

The "target cells" are those cells to be
10 mutagenized. The target cells specifically disclosed
herein are mouse embryonic stem cells. However, other cells
can be utilized. The "gene-targeted cells" are those cells
which are mutagenized. Gene-targeted cells lacking at least
one normal copy of SOD-1 allele can be utilized in the
15 generation of gene-targeted mice, for example. The gene-
targeted cells which lack at least one normal SOD-1 allele
can be utilized, for example, in high through-put screening
of agents such as superoxide scavenging compounds. Such
"screening cells" can be cryopreserved until utilized for
20 such screening. See, for example, Oncea et al., *Int.
Immunol.*, 6: 1161-1168, 1994 and Arbones et al., *Nat.
Genet.*, 6: 90-97, 1994.

The specific procedure followed is detailed below.
The following restriction enzymes, and their single letter
25 codes, are referred to in the examples which follow: EcoRI
(E), HindIII (H), KpnI (K), EcoRV (R), SalI (S) and NotI
(N).

Example 1
Cloning of Mouse SOD-1

30   The mouse SOD-1 genomic DNA was cloned from a phage
library created from 129/Sv mouse DNA partially digested
with *Sau*3A and inserted into the *Bam*HI site of Lambda DASH$^{®}$II
(Reaume et al., *Science, in press*, 1995). Using standard
molecular biology techniques (Maniatis et al., <u>Molecular</u>
35 <u>Cloning: A Laboratory Manual</u>, Cold Spring Harbor Laboratory,
Cold Spring Harbor, New York, 1982) approximately 1.2 x 10^6

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recombinant bacteriophages were screened for the presence of
SOD-1 sequences by hybridization with a 900 base pair (bp),
radiolabelled SOD-1 intron-specific DNA probe. This 900 bp
probe was generated by polymerase chain reaction (PCR)
5   amplification (Mullis and Faloona, Methods in Enzymolology,
Vol. 155, pp. 335-350, 1987, incorporated herein by
reference) of mouse genomic DNA using primers EH100 (ACC GGA
ATT CCA TAT AAG GAT ATA TAC A; SEQ ID NO:1) and EH101 (TAG
CGA ATT CAG GTT TGA ATG ATC AAG T; SEQ ID NO:2) which
10   hybridize to each end of the SOD-1 intron 4 (Figure 2b).
The approximate placement of the 5 SOD-1 exons is shown in
Figure 2b as based on data from Bendetto et al., Gene, 99:
191-195, 1991, incorporated herein by reference, and marked
above the map with bold numbers. The corresponding introns
15   are numbered below the map in bold italics.

The amplified fragment was separated from the other
components of the reaction by electrophoresis on a 1.0%
agarose gel, and purified using GeneClean II (Bio 101, Inc.,
La Jolla, CA). Purified probe DNA was radioactively
20   labelled with ^{32}P-dCTP by the random primer method using
materials and methods supplied by the kit manufacturer
(Multiprime DNA Labeling System; Amersham Life Sciences,
Arlington Heights, IL).

From this screen, 9 clones were identified which
25   hybridized to the SOD-1 intron probe: λSOD12, λSOD72,
λSOD20I, λSOD18, λSOD69, λSOD20G, λSOD47, λSOD67, and λSOD65
(Figure 2a). These clones were purified by limiting
dilution and plaque hybridization with the SOD-1 intron 4
probe (Maniatis et al., 1982, supra).

30   For each clone, DNA was prepared from bacteriophage
particles first purified on a CsCl gradient (Maniatis et
al., 1982, supra). Restriction maps were then generated for
each of the cloned inserts using the FLASH° Nonradioactive
Gene Mapping Kit (Stratagene° Inc., La Jolla, CA), as
35   summarized in Figure 3. This method of restriction enzyme
mapping involves first completely. digesting 10 μg of the
phage DNA with the restriction enzyme NotI using standard

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restriction enzyme digest conditions (Maniatis et al.,
1982, *supra*). *Not*I cuts all clones in the vector DNA at
either end of the cloned insert, leaving a T3 bacteriophage
promoter attached to one end of the insert and a T7

5   bacteriophage promoter attached to the other end. The *Not*I
digested DNA was then partially digested with the enzyme
*Eco*RI, as an example, using limiting amounts of enzyme (0.2
units/µg DNA), in an 84 µl reaction volume at 37°C.
Aliquots (26 µl) were removed after 3 minutes, 12 minutes,

10   and 40 minutes and the digest reaction was stopped by the
addition of 1 µl of 0.5 M EDTA. DNA from all three time
points was resolved on a 0.7% agarose gel, visualized by
ethidium bromide staining, and then transferred to a
GeneScreen Plus° membrane (NEN° Research Products, Boston,

15   MA) by capillary transfer (Maniatis et al., 1982, *supra*).
The membrane was hybridized with an alkaline phosphatase
labelled oligonucleotide that was specific for the T3
promoter (supplied with the FLASH° kit) using reagents and
methods supplied by the kit manufacturer. After

20   hybridization, the membrane was washed and developed with a
chemiluminescent-yielding substrate and then exposed to X-
ray film in the dark for approximately 60 minutes.
        The oligonucleotide probes effectively label one
end of the insert. By determining the positions of the

25   bands on the X-ray film and calculating the DNA size to
which they correspond, it was possible to determine the
position of the *Eco*RI sites relative to the T3 end of the
insert. These results were then complemented by stripping
the probe off of the membrane, and rehybridizing with a T7-

30   specific oligonucleotide in order to determine the positions
of the *Eco*RI sites relative to the T7 end of the insert.
This process was repeated using the enzymes *Hind*III and
*Kpn*I.
        The results of restriction mapping of the 9

35   different SOD genomic clones using the FLASH® Nonradioactive
Gene Mapping Kit (Stratagene® Inc., La Jolla, CA) are
depicted in Figure 2a. Some clones were isolated multiple

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times and therefore have more than one name. By comparing
the restriction enzyme maps of the different overlapping
clones, a composite map was assembled (Figure 2b). Of the 9
original clones isolated, a total of six independent clones
5   were identified.

Example 2
Construction of a Deletion Vector
        Examination of the published report that describes
the structure of the mouse SOD-1 gene (Bendetto, et al.,
10 *supra*), revealed that the entire coding sequence of the
mouse SOD-1 gene is within a 7.2 kb *Eco*RI fragment of DNA
(Figure 2b). Based on available restriction site data and
preferred sizes for arms of homology (Deng et al., *Mol.*
Cell. Biol., 12: 3365-3371, 1992; Zhang et al., *Mol. Cell.*
15 *Biol.*, 14: 2402-2410, 1994), a 4.9 kb *Hind*III-*Eco*RI fragment
was selected for the 5'-arm of homology and a 3.3 kb *Kpn*I-
*Eco*RV fragment was selected for the 3' arm (Figure 4a). A
targeting vector was created by isolating these two
fragments and placing them into a plasmid which contained a
20 neo^r cassette (a neomycin phosphotransferase gene linked to a
phosphoglycerate kinase promoter) as a positive selection
marker, a TK cassette (a Herpes simplex virus thymidine
kinase gene linked to a phosphoglycerate kinase promoter) as
a negative selection marker, and linker sequences, to
25 produce the deletion vector pSOD-TV. The process is set
forth in detail below.
<u>Construction of the Intermediate Plasmid pPNTlox^2</u> pSOD-TV
was created from a derivative of pPNT (Tybulewicz, et al.,
Cell, 65: 1153-1163, 1991, incorporated herein by reference;
30 obtained from Dr. Richard Mulligan, MIT, Cambridge, MA) by
first inserting two oligonucleotide linkers on each side of
the neo^r cassette creating the intermediate plasmid pPNTlox^2
(Figure 5). A double-stranded 79 base pair 5' linker having
*Sal*I, *Hpa*I, and *Nsi*I sites was created by annealing two
35 single-stranded oligonucleotides that overlap at their 3'
ends and then filling in the remaining single-stranded

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regions with the Klenow fragment of DNA polymerase I.   The
oligonucleotides PNT Not (GGA AAG AAT GCG GCC GCT GTC GAC
GTT AAC ATG CAT ATA ACT TCG TAT; SEQ ID NO:3) and PNT Xho
(GCT CTC GAG ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TAT
5   GC; SEQ ID NO:4) (150 ng of each) were combined in a 30 μl
reaction mixture containing 5 U of Klenow polymerase, Klenow
polymerase buffer, and 2mM dNTPs (dATP, dCTP, dGTP, and
dTTP).   After incubating for 1 hour at 37°C, a portion
(5μl) of this reaction mixture was simultaneously digested
10   with the restriction enzymes *Not*I and *Xho*I to liberate the
restriction enzyme sites at each end of the linker.   In
addition, 200 ng of pPNT was digested with *Not*I and *Xho*I.
The digested plasmid was resolved on a 0.8% agarose gel,
purified from the gel, and treated with calf intestinal
15   phosphatase according to standard methods   (Maniatis et al.,
1982, *supra*).   A quantity (66 ng) of the double digested
linker was ligated to the double digested and phosphatase-
treated pPNT DNA   (Maniatis et al., 1982, *supra*).   Following
DNA transformation of competent WM1100 *E. coli* (Dower,
20   *Nucleic Acids Res.* 16: 6127-6145, 1988, incorporated herein
by reference), plasmid DNA was isolated from ampicillin-
resistant bacteria (Holmes et al., *Anal. Biochem.* 114: 193-
197, 1981, incorporated herein by reference) and analyzed by
restriction enzyme analysis.   The proper recombinant
25   plasmids were identified as having acquired *Sal*I, *Hpa*I, and
*Nsi*I sites while still retaining the *Not*I and *Xho*I sites of
the starting plasmid.   One such recombinant plasmid with a
79 bp linker sequence was identified and designated pXN-4
(Figure 5).
30   A similar approach was used to insert a 40 bp 3'
linker between the *Xba*I and *Bam*HI sites of pXN-4.   The
oligonucleotides used to synthesize the linker were PNT Xba
(CGT TCT AGA ATA ACT TCG TAT AAT GTA TGC TAT; SEQ ID NO:5)
and PNT Bam (CGT GGA TCC ATA ACT TCG TAT AGC ATA CAT TAT;
35   SEQ ID NO:6).   Plasmid pXN-4 and the double-stranded linker
DNA were digested with *Xba*I and *Bam*HI.   The purified
fragments were joined by DNA ligation and transformed into

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competent WM1100 *E. coli* bacteria. Plasmid DNA was digested with *Xba*I and *Bam*HI, end-labelled with ^{32}P-dCTP and Klenow polymerase, and resolved on an 8% acrylamide gel (Maniatis et al., 1982, *supra*). The gel was dried and exposed to X-

5   ray film. Proper recombinant clones were identified by the presence of a 40 bp band liberated by the *Xba*I-*Bam*HI double digest. The resulting plasmid was called pPNTlox^2 (Figure 5). This construct includes the neo^r flanked by the loxP sequences; see Sauer, *supra*.

10   To confirm the sequences of the inserted linkers, a fragment containing both linkers was isolated from pPNTlox^2, using *Not*I and *Eco*RI, and cloned into pBlueScript$^•$SK+, a vector more amenable to nucleotide sequencing. Identity of the linkers was confirmed by direct nucleotide sequencing

15   (Sanger, *Proc. Natl. Acad. Sci. USA*, 74: 5463-5467, 1977) using T3 and T7 sequencing primers (Stratagene$^•$ Inc., La Jolla, CA).

Construction of the pSOD-TV deletion vector: The deletion vector pSOD-TV was assembled by inserting the selected 5'

20   and 3' arms of homology appropriately into pPNTlox^2. Initially, both arms of homology were subcloned from the phage inserts into pBlueScript$^•$ SK+. The map of λSOD18 (Figure 2a) shows restriction enzymes sites for *Kpn*I (K), *Eco*RI (E), *Hind*III (H), and *Sal*I (S). The T3 and T7

25   promoters at either end of the cloned inserts are indicated. The 3' arm of homology was isolated from λSOD18 by digesting 10 μg of bacteriophage DNA with the enzymes *Kpn*I and *Sal*I, resolving the digested DNA on a 0.8% agarose gel, and purifying the excised 3.3 kb fragment with GeneClean$^•$ II (Bio

30   101 Inc., La Jolla, CA). The same digest and gel isolation procedure were performed in parallel with pBlueScript$^•$SK+ DNA except that the purified band was 3.0 kb. Approximately 400 ng of the purified lambda DNA and 100 ng of the purified plasmid DNA were combined in a 10 μl ligation reaction.

35   Following transformation of competent WM1100 *E. coli*, plasmid DNA was isolated from ampicillin-resistant bacteria and analyzed by restriction enzyme analysis to identify the

resultant plasmid pSK18-9 (Figure 6).

To clone the 3' arm of homology into pPNTlox^2 , the arm was liberated from pSK18-9 by enzymatic restriction with *Kpn*I and *Eco*RV and purified by gel isolation. The plasmid
5   pPNTlox^2 was digested with *Eco*RI and the resultant 4 base overhang was filled-in using Klenow polymerase (Maniatis et al., 1982, *supra*). Following further digestion with *Kpn*I, the pPNTlox^2 plasmid DNA was gel purified and ligated to the purified 3' arm of homology. Following bacterial
10 transformation, proper recombinants were identified by restriction enzyme analyses. The resulting plasmid was designated pSOD3'homolTV (Figure 7).

A similar approach was used to isolate, purify, and subclone the 5' arm of homology into pBlueScript$^\bullet$ SK+. The
15 map of λSOD69 (Figure 2a) shows restriction enzyme sites for *Kpn*I (K), *Eco*RI (E), and *Hind*III (H). The T3 and T7 promoters at either end of the cloned insert are indicated. The 5' arm was isolated from λSOD69 by first digesting the bacteriophage DNA with *Hind*III, isolating an 11.8 kb DNA
20 fragment by gel electrophoresis, and then partially digesting this DNA fragment with limited amounts of *Eco*RI (1U/μg) for 1 to 5 minutes. The reaction was stopped with 20 mM EDTA. A 4.9 kb DNA fragment was purified after agarose gel electrophoresis and cloned into the *Eco*RI and *Hind*III
25 sites of pBlueScript$^\bullet$ SK+ to generate pSK EH69-2 (Figure 8).

To construct plasmid pSOD-TV, the 5' arm of homology was removed from pSKEH69-2 by first digesting with *Not*I, filling-in the overhang with Klenow polymerase, and then digesting with *Sal*I. In parallel, pSOD3'homolTV was
30 first partially digested with *Hpa*I, then completely digested with *Sal*I. The final deletion vector pSOD-TV was constructed by ligation of the two gel-purified fragments (Figure 9).

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Exampl   3

D l tion of th   SOD-1 Gene in ES cells

Cells: The R1 line of ES cells derived from 129/Sv x
129/Sv-CP F1 hybrid mice (pigmented) (Nagy, et al., *Proc.*
5   *Natl. Acad. Sci. USA*, 90: 8424-8428, 1993, incorporated
herein by reference) was obtained from Dr. Janet Rossant,
Dr. Andras Nagy, Reka Nagy, and Dr. Wanda Abramow-Newerly
(Mt. Sinai Hospital, Toronto, Ontario, Canada).   The cells
were grown in ES cell medium consisting of Dulbecco's
10   Modification of Eagle's Medium (Eagle's medium containing L-
glutamine and 4500 mg/L glucose; Mediatech Inc., Herndon,
VA) supplemented with   20%   fetal bovine serum (FBS; Hyclone
Laboratories Inc., Logan, Utah; cat. # A-1115; Lot #
11152154),   0.1 mM non-essential amino acids (Mediatech 25-
15   025-L1), 2 mM L-glutamine (Mediatech 25-005-L1), 10^{-6} M β-
mercaptoethanol (Gibco 21985-023), 1 mM sodium pyruvate
(Mediatech 25-000-L1), 1x concentration of a penicillin (50
IU/ml) streptomycin (50 mcg/ml) solution (Mediatech 30-001-
L1), and 1000 U/ml of leukemia inhibitory factor   (Gibco BRL
20   13275-029).   The cells were grown on tissue culture plastic
that had been briefly treated with a solution of 0.1%
gelatin (Sigma G9391), i.e., gelatinized plates.

The cultures were plated at 1 X 10^5 cells per ml in
100 mm X 15 mm plastic culture plates and passaged every 48
25   hours, or when the cells became about 80% confluent.   For
passage, the cells were first washed with phosphate buffered
saline without Ca^{2+} and Mg^{2+}, hereinafter referred to as
"PBS", and then treated with a trypsin/EDTA solution (.05%
trypsin, .02% EDTA in PBS).   After all of the   cells were in
30   suspension, the trypsin digestion was stopped by the
addition of ES cell medium.   The cells were collected by
centrifugation, resuspended in 5 ml of ES cell medium, and a
1 ml aliquot of the cell suspension was used to start a new
plate of the same size.
35   DNA Gene-Targeting of ES cells: pSOD-TV DNA (400 µg) was
prepared for electroporation by digesting it with Not I in a
1 ml reaction volume.   The DNA was then precipitated by the

addition of ethanol, washed with 70% ethanol, and
resuspended in 500 μl of sterile water.

    The *Not*I-linearized pSOD-TV DNA was electroporated
into ES cells using a Bio-Rad Gene Pulser® System (Bio-Rad
5 Laboratories, Hercules, CA) as follows. In each of 10
electroporation cuvettes, 40 μg of DNA was electroporated
into 5×10^6 cells suspended in 0.8 ml ES cell medium. The
electroporation conditions were 250 V and 500 μF which
typically result in time constants ranging between 5.7 - 6.2
10 seconds. After electroporation the cells were incubated for
20 minutes at room temperature in the electroporation
cuvettes. All the electroporated cells were then pooled and
distributed approximately equally onto 20 gelatinized plates
(100 mm X 15 mm).

15   After 24 hours, the plates were aspirated and fresh
ES cell medium was added. The following day, the medium in
19 plates was replaced with ES cell medium supplemented with
150 μg/mL of G418 (Gibco) and 0.2 μM ganciclovir (Syntex,
Palo Alto, CA). The medium in one plate was supplemented
20 with 150 μg/mL of G418 alone. After an additional 6 days,
resultant individual ES cell colonies were picked off of the
plates and separately expanded in individual wells of 24
well plates as described by Wurst et al., Gene Targeting
Vol. 126, Edited by A. L. Joyner, IRL Press, Oxford
25 University Press, Oxford, England, pp. 33-61, 1993,
incorporated herein by reference. A comparison of the
number of colonies that grew on the plates supplemented with
G418 and ganciclovir versus the number that grew on the
plates supplemented G418 alone was used to determine the
30 efficiency of negative selection, which was 3.2 fold.
Analyses of the gene-targeted ES cells: When the cell
culture in each well of the 24-well plates became
approximately 80% confluent, the cells were washed with PBS
and then dispersed with two drops of trypsin-EDTA.
35 Trypsinization was stopped by the addition of 1 ml of ES
cell medium. An aliquot (0.5 mL) of this suspension was
transferred to each of two wells of separate 24-well plates.

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After the cells had grown to near confluence, one of the
plates was used for cryopreservation of the cell line while
the other was used as a source of DNA for each of the cloned
cell lines.

5   For cryopreservation, the cells in a 24-well plate
were first chilled by placing the plate on ice. The medium
was then replaced with fresh ES cell medium supplemented
with 10% DMSO and 25% FBS. The plate was then cooled at
approximately 0.5°C minute by insulating the plate in a
10   styrofoam box and placing it in a -70°C freezer.

To isolate the DNA from the cloned cell lines on
the other 24-well plate, the medium in each well was
replaced with 500 μl of digestion buffer (100 mM Tris-HCl,
pH8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg/ml
15   proteinase K) and incubated overnight at 37°C. After
overnight incubation, 500 μl of isopropanol was added to
each well and the plate was agitated for 15 minutes on an
orbital shaker. The supernatant fluid was aspirated and
replaced with 500 μl of 70% ethanol and the plate was shaken
20   for an additional 15 minutes. The DNA precipitate was
picked out of the well and dissolved in 50 μl of TE solution
(10 mM Tris-HCl pH 7.5, 1 mM EDTA).

The primary analysis for deletion of the SOD-1 gene
involved a Southern hybridization screen of ApaI digested ES
25   cell DNA. The probe for this analysis was derived from the
5' end of the SOD gene outside of the 5' arm of homology
(Figure 10a). An aliquot (10 μl) of each DNA sample was
digested with ApaI, resolved on a 0.8% agarose gel, and
transferred to a GeneScreen Plus® membrane. The probe was
30   prepared by first isolating the 1.3 kb EcoRI-HindIII
fragment from λSOD69 (Figure 2a). Subsequent AluI digest of
this fragment yielded the 600 base pair probe. The probe
was labelled with ^{32}P-dCTP by random priming and hybridized
overnight to the membrane at 58°C (Church et al., Proc.
35   Natl. Acad. Sci. USA, 81: 1991-1995, 1984). An ES cell
line in which the SOD-1 gene has been successfully deleted
yields 9 kb and 10 kb ApaI fragments, in this assay (Figure

10). The targeting event replaces all of the SOD-1 coding
sequence and introns with the neo^r positive selection marker.
A normal SOD-1 gene carries an ApaI site located
approximately 1.0 kb downstream from the 5' border of the
5 region to be deleted. The neo^r marker which replaces the
SOD-1 gene carries with it an *ApaI* site near its 5' end. As
a result, this assay yields a 10 kb *ApaI* fragment from the
normal SOD-1 gene and a 9 kb *ApaI* fragment from the deleted
SOD-1 gene.

10   All cell lines scored as putative homologous
recombinants by the primary screen were then further
screened using a 1.8 kb *EcoRI* probe (isolated from an
λSOD69, *EcoRI* digest) on *SpeI* digested ES cell DNA. In
this case, the normal SOD-1 gene yielded a 9 kb fragment and
15 the mutant SOD-1 gene a 10 kb fragment (Figure 10.) From 80
cell lines (numbered 1-80) whose DNA was analyzed, five were
identified as having undergone proper homologous
recombination. Three of the five cell lines that were
identified as having undergone proper homologous
20 recombination by both screens were then thawed and their
cell numbers expanded. Cells from the resulting cultures
were used to make chimeric mice.

Example 4

Establishment of SOD-1 Null Mice
25   SOD-1 gene-targeted ES cells were used to make
chimeric mice by aggregating the ES cells to E2.5 embryos
and transferring the aggregated embryos to pseudopregnant
females. (Wood et al., *Nature*, 365: 87-89, 1993,
incorporated herein by reference). ES cells were prepared
30 for aggregation by limited trypsinization to produce clumps
that averaged 10-15 cells. E2.5 embryos were collected
from superovulated CD-1 female mice (albino) by oviduct
flushing as described by Hogan et al., <u>Manipulating the
Mouse Embryo: A Laboratory Manual</u>, Cold Spring Harbor
35 Laboratory, Cold Spring Harbor, New York, 1986). The zona
pellucida was removed from the embryos using acidic Tyrode's

solution (Sigma Chemical Co., St. Louis, MO). Aggregation
wells were created by pressing a blunt metal instrument
i.e., a darning needle into tissue culture plastic.
Embryos were then placed in a well together with a clump of
5   approximately 10-15 ES cells in a small drop (approximately
20 μl) of M16 medium (Sigma Chemical Co., St. Louis, MO)
under mineral oil.   After an overnight incubation (37°C,
100% humidity, 5% CO_2 in air), approximately 20 of the
aggregated embryos were transferred to the uterine horns of
10   each pseudopregnant female (Hogan et al., *supra*).
Contribution of the ES cells to the offspring was scored by
the appearance of pigmented coat color. Pigmented mice were
termed chimeric founders. Germline contribution by the ES
cells was scored by the appearance of pigmented offspring
15   from a cross between the chimeric founders and CD-1 females.
        Three of the five gene-targeted ES cell lines were
used in embryo aggregations. One (line 42) gave five
germline chimeras, another (line 58) gave four germline
chimeras, and the third (line 66) gave none (See Table 1).

20   **Table 1**

Cell Line Number	Number of Embryo Aggregations	Number of Pups Born	Number of Chimeric Founders	Number of Germline Chimeras
42	138	31	18	5
58	130	24	14	4
66	141	33	3	0

The germline chimeras were then used to establish
lines of SOD-1 deficient mice and mice lacking SOD-1. The
presence of the gene-targeted SOD-1 allele in the pigmented
offspring was determined using the Southern blot strategy
30   described above with genomic DNA prepared from a tail sample
(Hogan et al., *supra*). Heterozygous SOD-1 null mice have 9
kb and 10 kb *Apa*I fragments that hybridize with the 5' *Alu*I
SOD probe. Homozygous SOD-1 null mice were established by

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crossing 2 heterozygous SOD-1 null mice and were identified
as having only a 9 kb *ApaI* genomic DNA fragment that
hybridized with the 5' *AluI* SOD probe.

Example 5
5   **Cu/Zn SOD Protein Levels and Enzymatic Activity in the SOD-1
Null Mice**
        To confirm that the targeted disruption of the SOD-1
locus results in a reduction of Cu/Zn SOD levels in the
tissues of the resulting mammals, blood samples were
10 collected from wild-type mice, and mice shown to be
heterozygous and homozygous for the SOD-1 gene. The blood
samples were analyzed for Cu/Zn SOD protein by immunoblot
analysis. Red blood cell lysates were prepared by lysing
the blood cells (approximately 75 ul) by several cycles of
15 alternately freezing and thawing. The protein
concentrations of the cell lysates were determined using the
BCA method (Pierce, Rockville, IL). An aliquot (2 to 2.5 μg
of protein) of each sample was electrophoresed on a 4-20%
polyacrylamide gel (Novex, San Diego, CA) using a
20 Tris/glycine/SDS (25 mM Tris/ 192 mM glycine/ 0.1%SDS)
buffer system.
        The separated proteins were transferred to
nitrocellulose filters by electroelution and the resulting
filters were blocked by incubation in blotto solution -- 5%
25 non-fat, dry milk in 25 mM Tris-buffered saline (1x TBS) --
for 30 minutes. The filters were then submersed in a
primary antibody solution (1:10,000 dilution in blotto
solution) and incubated for between 2 and 18 hours. The
primary antibody used was polyclonal rabbit antisera raised
30 against purified mouse Cu/Zn SOD protein produced in *E. coli*
(Hazelton Research Products, Denver, PA). The filters were
washed three times for 5 minutes each in 1x TBS and
incubated in secondary antibody solution (1:2,000 dilution
in blotto solution) for two hours. The secondary antibody
35 was a goat anti-rabbit IgG conjugated to alkaline
phosphatase (Bio-Rad, Richmond, CA). The filters were

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washed three times for 5 minutes each in 1x TBS and stained
for alkaline phosphatase activity by incubating them for
between 5 and 60 minutes in a commercially available
alkaline phosphatase detection reagent (Bio-Rad, Richmond,
5   CA).

Stained bands corresponding to Cu/Zn SOD protein were
quantitated using a DocuGel V image analysis system and
RFLPscan software (Scanalytics, Billerica, MA). The levels
of Cu/Zn SOD protein are depicted in Figure 11 (solid bars)
10   and are expressed relative to the level of Cu/Zn SOD protein
in the samples from wild-type mammals. The results of these
studies indicate that the heterozygous SOD-1 null mice
exhibit an expected near 50% reduction in Cu/Zn SOD protein.
Further, the homozygous SOD-1 null mammals showed no
15   detectable Cu/Zn SOD protein on Western blots.

Cu/Zn SOD enzymatic activity in the red blood cell
lysates was measured using the NADPH oxidation method of
Paoletti, et al. (*Anal. Biochem.*, 154:536-541, 1986,
incorporated herein by reference). Protein samples (1 mg)
20   from the wild-type, heterozygous SOD-1 null mice, and
homozygous SOD-1 null mice were extracted with an equal
volume of ethanol:chloroform (2:1) and the resulting
supernatant fluid was dialyzed overnight against PBS saline
at 4°C. The protein concentration of the dialysate was
25   determined using the BCA method (Pierce, Rockville, IL) and
10 ug of each sample was assayed for SOD activity.
Enzymatic activity was expressed relative to the wild-type
control sample (Figure 11, cross-hatched bars). The Cu/Zn
SOD activity of the sample from heterozygous SOD-1 null
30   mammals was approximately 50% of that displayed by samples
from the wild-type mammals, consistent with the expectation
that only one SOD-1 allele was active in the heterozygous
mammals. Furthermore, the protein sample derived from the
homozygous SOD-1 null mice showed nearly a total reduction
35   in Cu/Zn SOD activity. The small residual activity detected
in this assay likely represents background activity
associated with the assay, but could also reflect an

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endogenous superoxide scavenging activity supplied by an
alternate protein.

Example 6

Maintenance conditions of SOD-1 Null Mice

5   Young adult heterozygous and homozygous SOD-1 null mice
are currently being maintained under viral and antigen free
conditions as defined by Charles River Laboratories,
Wilmington, MA. Presently, the diet for these mice is the
same as that provided to laboratory mice. No other unique
10 or distinguishing living conditions have thus far been
required for the mice.

       Attempts at the breeding of homozygote males with
homozygote females have been unsuccessful thus far, which we
believe is due to the deficiency of Cu/Zn SOD and the
15 oxidative stress occasioned by pregnancy. The homozygote
breeding pairs are capable of conceiving offspring, but the
litters have been small in number (1-2) and the pups are
either still-born, or those pups which are born alive die
almost immediately.

20   The colony can be propagated by the breeding of
heterozygous SOD-1 null males and females, homozygous SOD-1
null males to either wild type or heterozygous SOD-1 null
females, or wild type males to homozygous SOD-1 null
females.

25   While the invention has been described and illustrated
with respect to specific embodiments, it is to be understood
that modifications and equivalents apparent to those skilled
in the art are intended to be within the scope of the
disclosure and the following claims.

30   All references referred to herein are hereby
incorporated by reference.

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What is claim d is:

1.   A heterozygous SOD-1 null mouse.

2.   A homozygous SOD-1 null mouse.

3.   A gene-targeted mouse lacking at least one normal SOD-1
5   allele.

4.   The mouse of claim 3 substantially lacking both normal
SOD-1 alleles.

5.   A gene-targeted mouse having a phenotype characterized
by a reduced amount of Cu/Zn SOD protein expression.

10   6.   The gene-targeted mouse of claim 5 wherein
substantially no measurable amount of Cu/Zn SOD is produced
by said mouse.

7.   A recombinant DNA construct comprising a first sequence
homologous to a sequence from the 5' flanking region of
15   genomic SOD-1 from a mammal, a second sequence homologous to
a sequence from the 3' flanking region of genomic SOD-1 from
said mammal, and a positive selection marker.

8.   The DNA construct of claim 7 further comprising a
negative selection marker.

20   9.   The DNA construct of claim 8 wherein said first
sequence is about 4.9 kb and said second sequence is about
3.3 kb.

10.   The DNA construct of claim 7 further wherein said first
and second sequences are substantially isogenic to genomic
25   SOD-1 of a select cell from said mammal.

11.   The DNA construct of claim 10 wherein said cell is an embryonic stem cell.

12.   A method for assessing the effectiveness of a compound for counteracting the deleterious effects of oxidative
5   stress comprising:
        a)   exposing a heterozygous or homozygous SOD-1 null gene-targeted mouse to oxidative stress;
        b)   administering said compound to said mouse; and
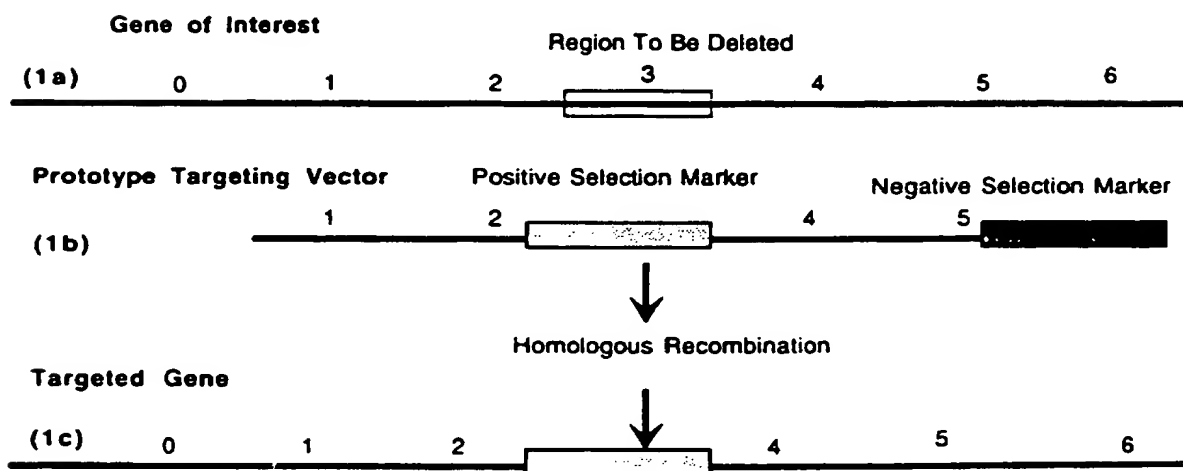   c)   comparing results obtained with said compound to a
10   control.

13.   A method for assessing the effectiveness of a compound for counteracting the deleterious effects of oxidative stress comprising:
        a)   exposing cells from a heterozygous or homozygous
15   SOD-1 null gene-targeted mouse to oxidative stress;
        b)   administering said compound to said cells; and
        c)   comparing results obtained with said compound to a control.

14.   A gene-targeted non-human mammalian cell lacking at
20   least one normal SOD-1 allele.

15.   The cell of claim 14 wherein said cell is an embryonic stem cell.

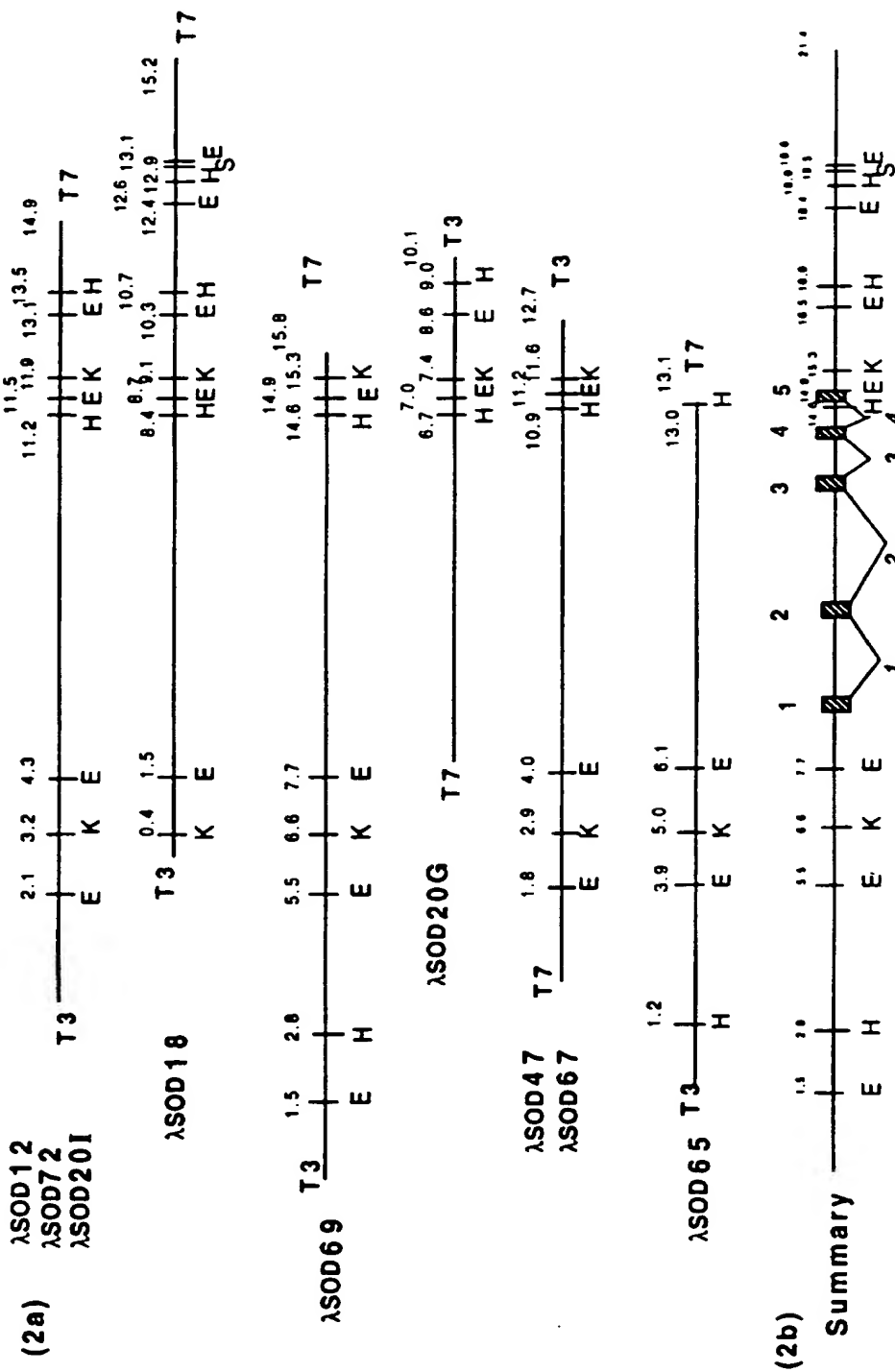16.   The cell of claim 15 wherein said embryonic stem cell is obtained from a mouse.

Figure 1

Prototype Gene Targeting Strategy

Figure 2

Mouse SOD-1 Genomic Clone Maps

(2a)

λSOD12
λSOD72
λSOD20I

λSOD18

λSOD69

λSOD20G

λSOD47
λSOD67

λSOD65

(2b) Summary

Restriction Mapping With FLASH™ G ne Mapping Kit

Figure 3
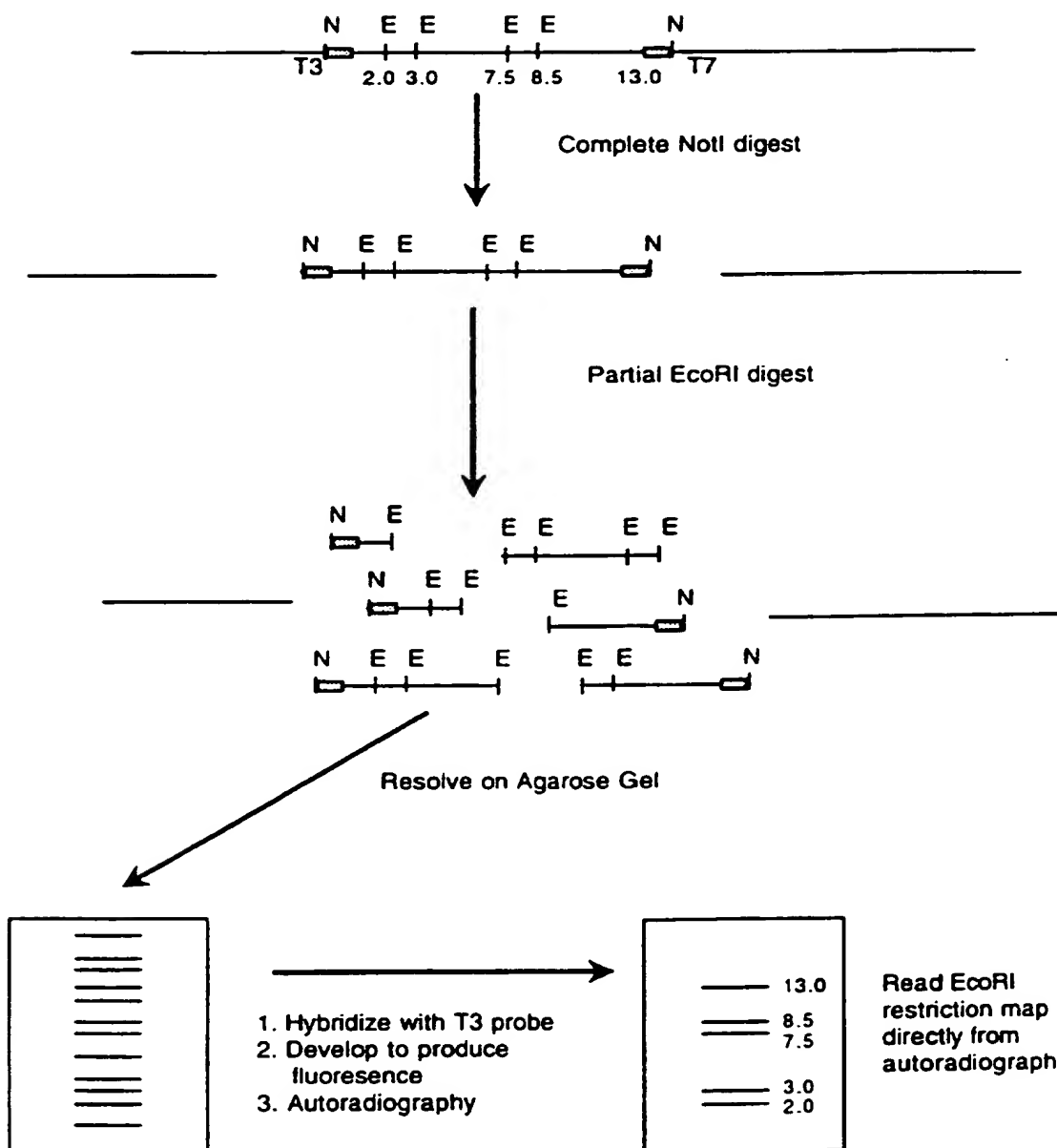
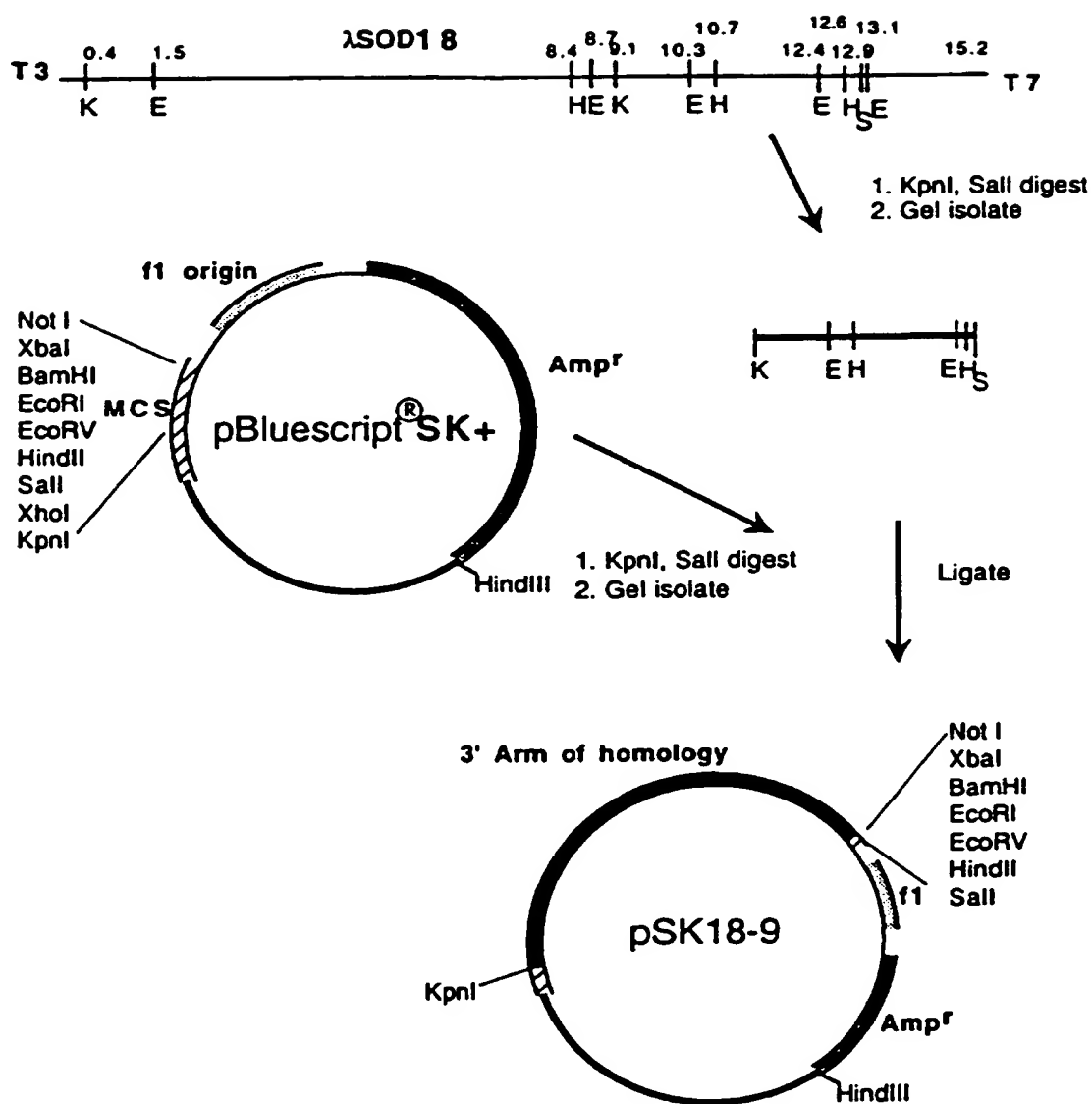Figure 4

SOD Genomic Map & pSOD-TV Deletion Vector

(4a)

(4b)

5' arm of homology

3' arm of homology

plasmid backbone

<u>Legend</u>

EcoRI - E
HindIII - H
KpnI - K
EcoRV - R
Sal I - S
Not I - N
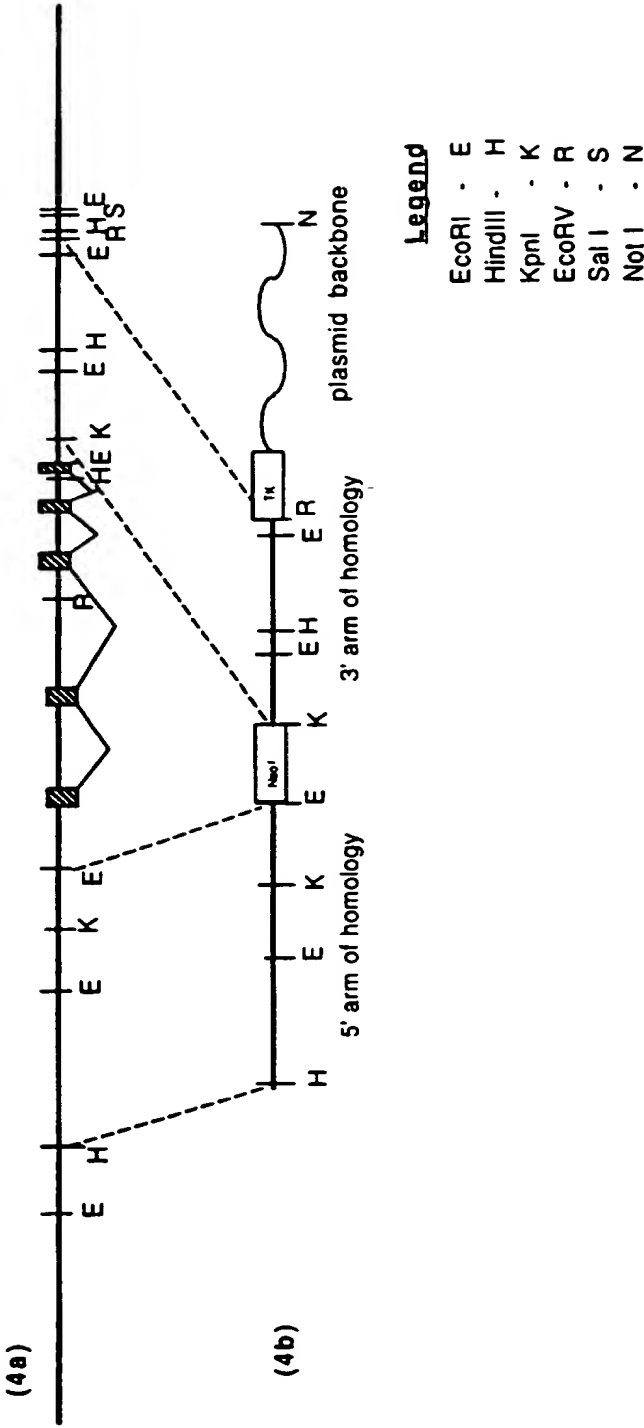
5/11

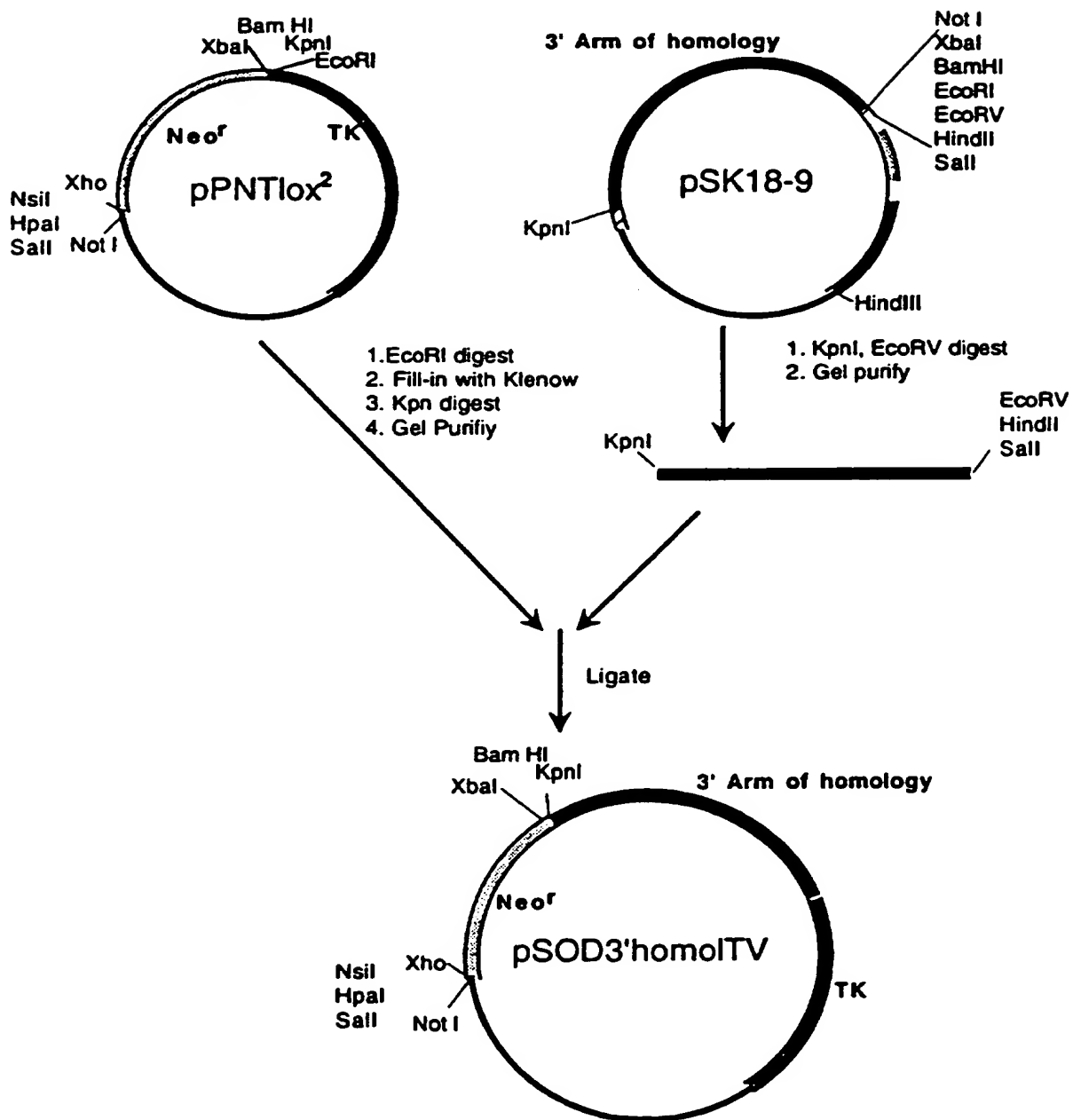## pPNTlox^2 Synthesis

<u>Figure 5</u>
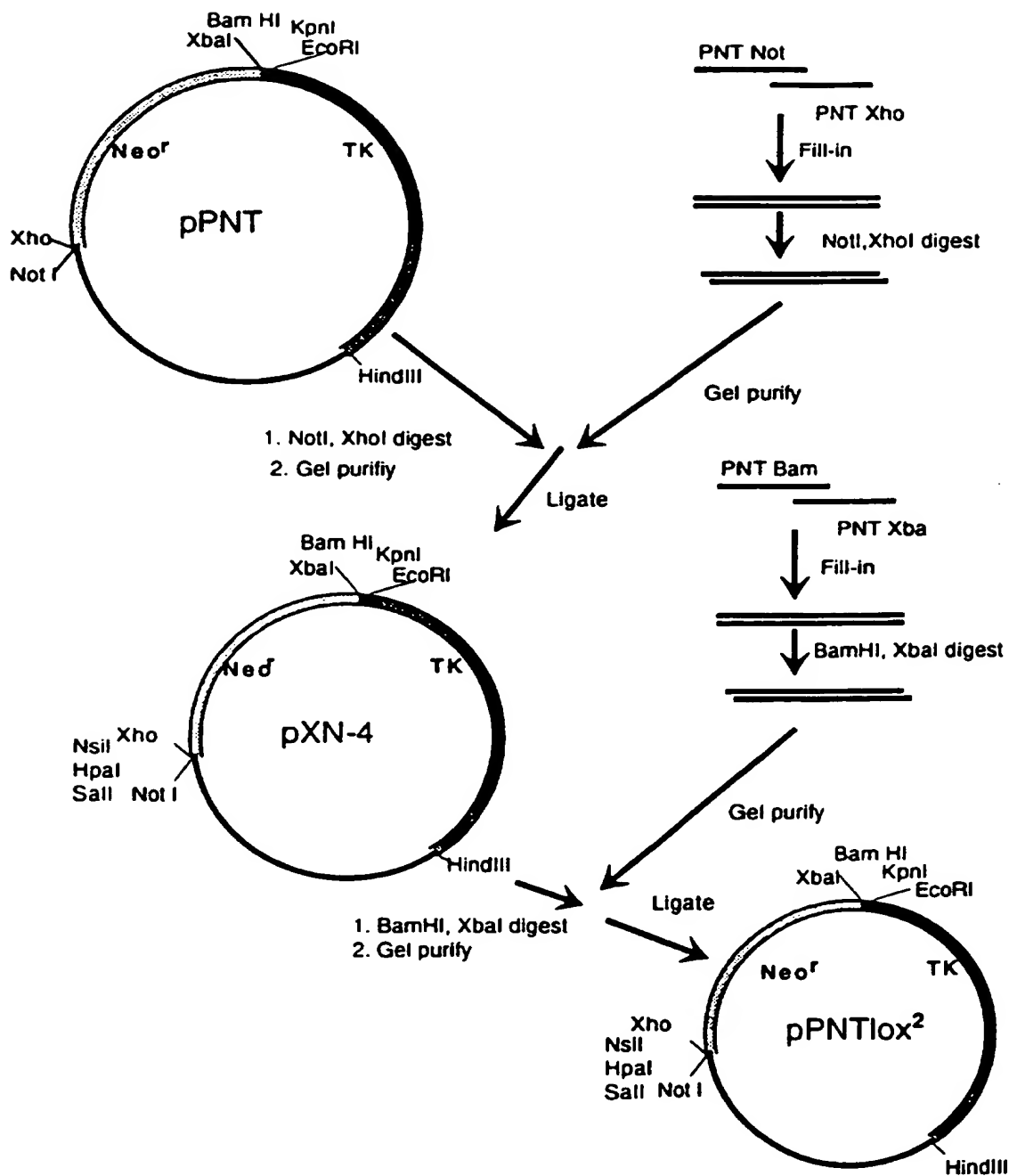
6/11

pKS18-9 Construction

Figure 6

·7/11

pSOD3'HomolTV Construction

<u>Figure 7</u>

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Figure 8   pSK EH69-2 Construction

λSOD6 9

5' Arm of homology
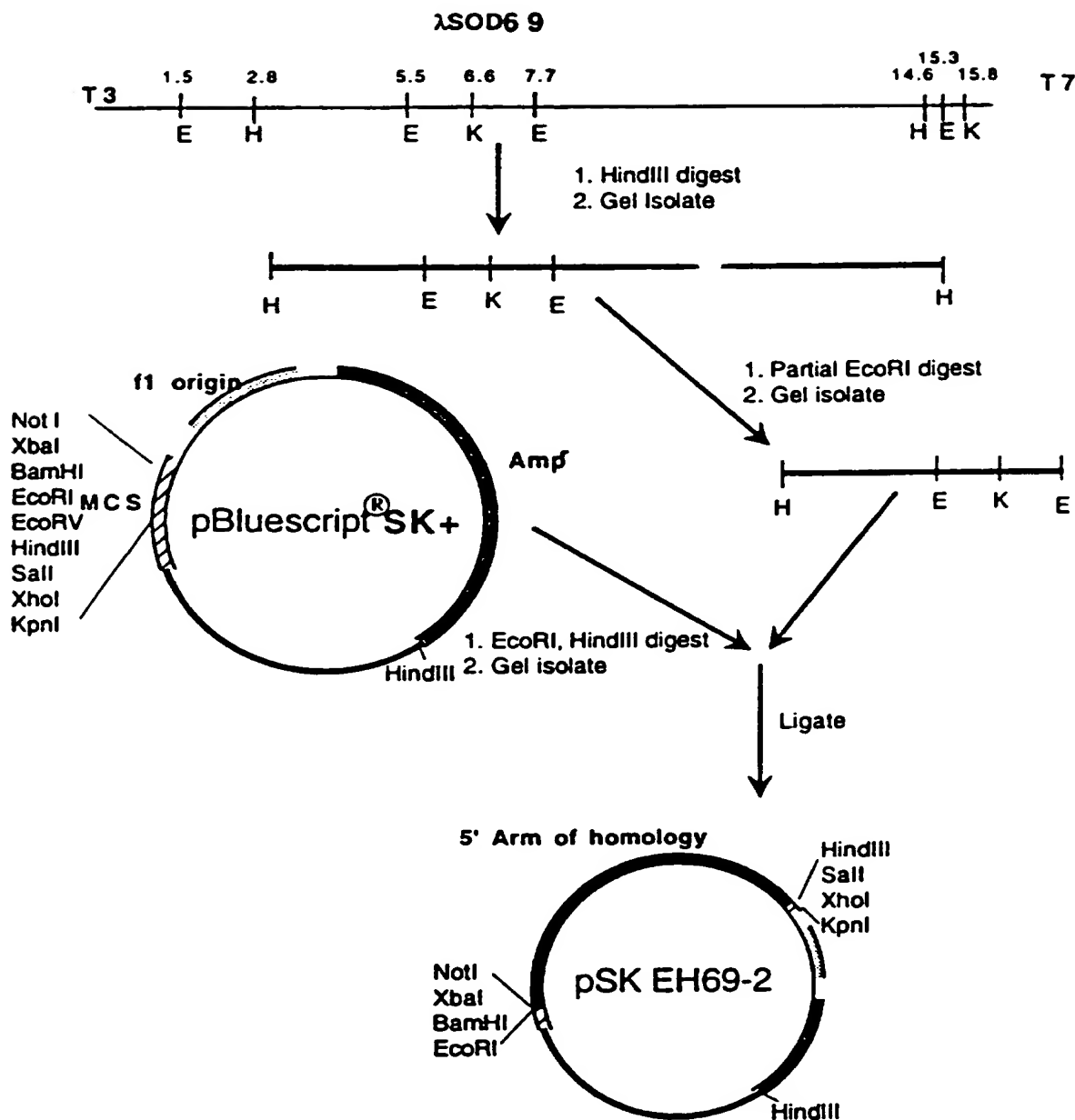
9/11

pSOD-TV Construction

<u>Figure 9</u>
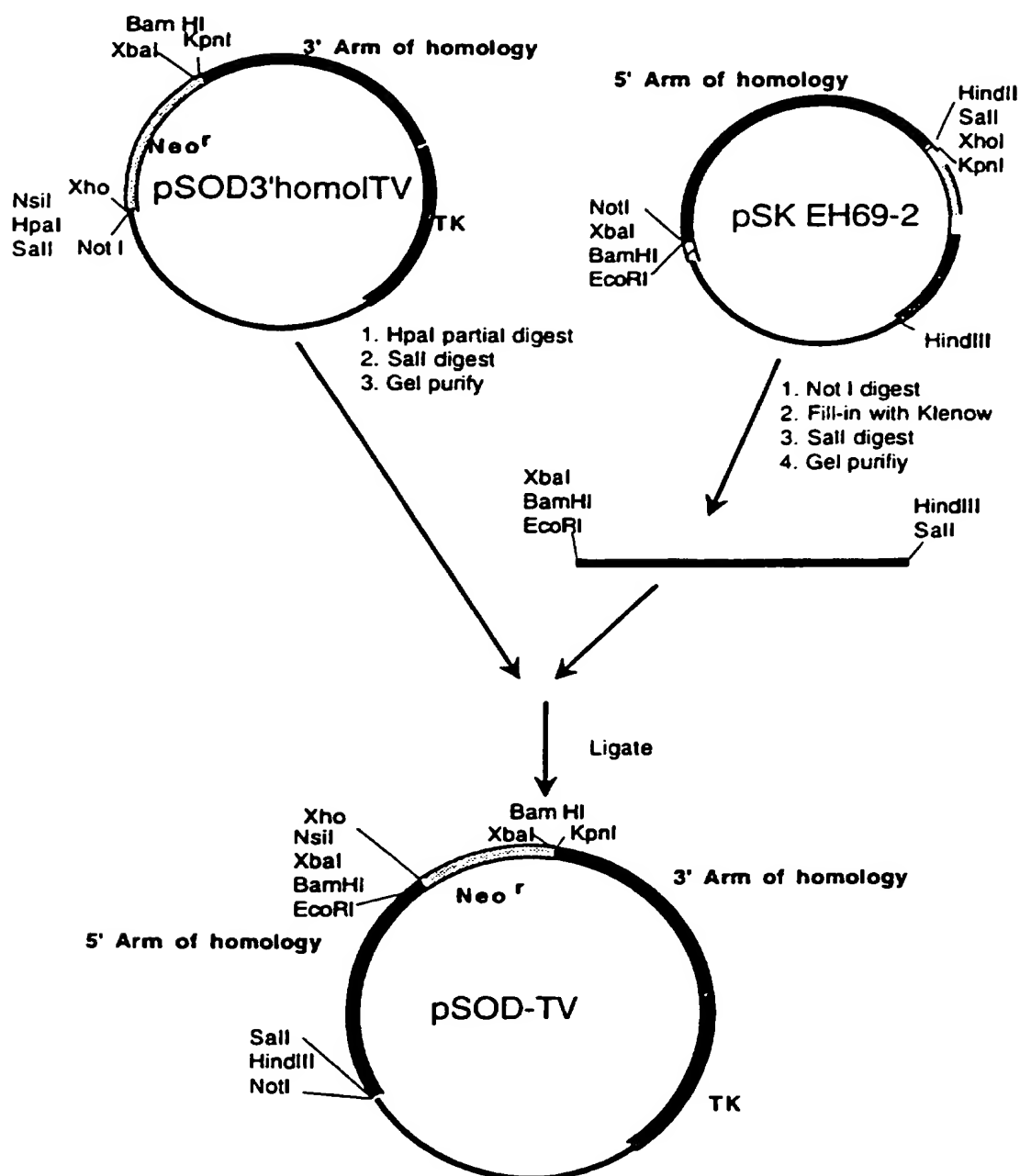
Figure 10

Strategy to detect homolologous recombination within mouse SOD-1
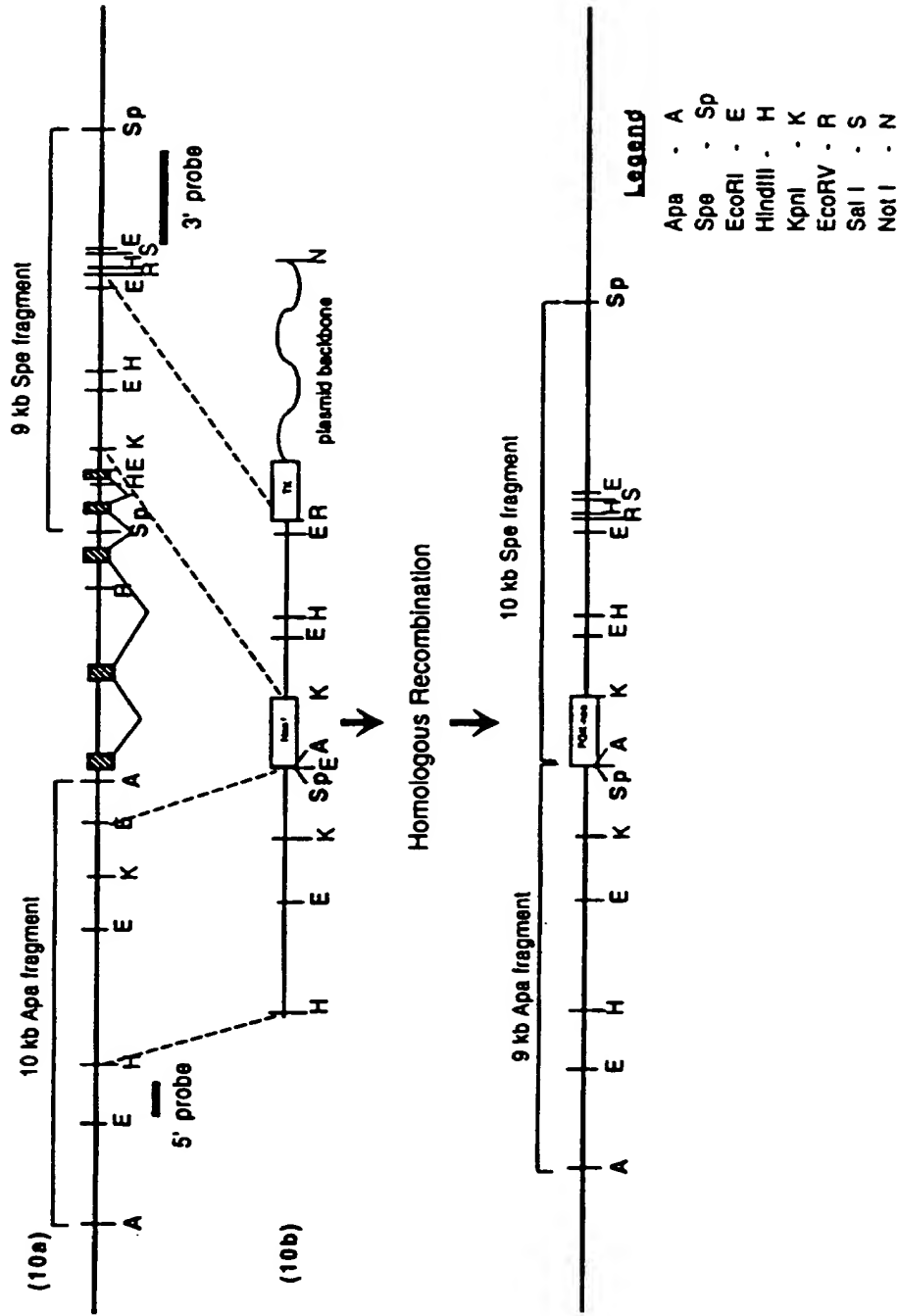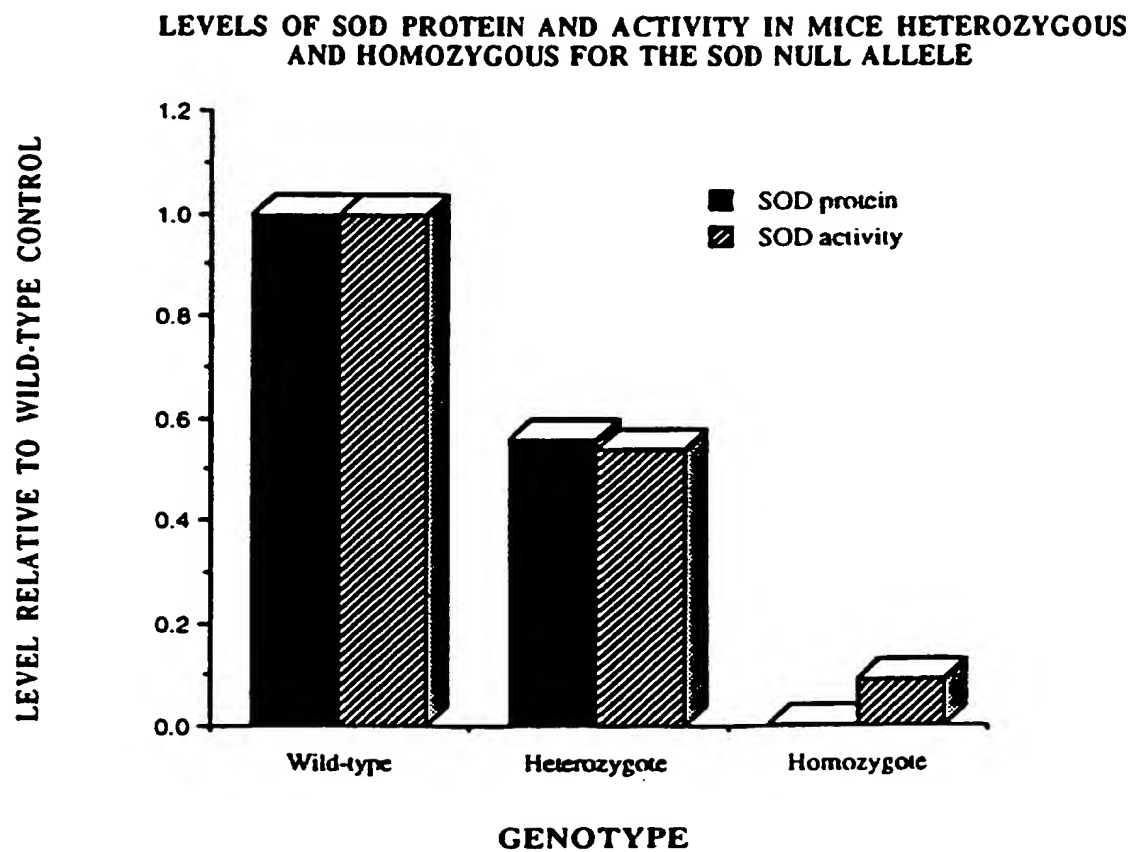
Legend

Apa · A
Spe · Sp
EcoRI · E
HindIII · H
KpnI · K
EcoRV · R
Sal I · S
Not I · N

FIGURE 11

LEVELS OF SOD PROTEIN AND ACTIVITY IN MICE HETEROZYGOUS
AND HOMOZYGOUS FOR THE SOD NULL ALLELE

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :A61K 49/00; C07H21/04; C12N 5/06, 15/06, 15/07

US CL   :424/9.1, 9.2; 435/172.3, 240.2, 240.21, 320.1; 536/24.1; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   424/9.1, 9.2; 435/172.3, 240.2, 240.21, 320.1; 536/24.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRADLEY, A. et al. Modifying the mouse: Design and Desire. Biotechnology. May 1992, Volume 10, pages 534-539, see entire article.	1-16
Y	BOWLING, A.C. Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. Journal of Neurochemistry. 1993, Volume 61, No. 6, pages 2322-2325, see entire article.	1-16

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 JUNE 1996	1 2 JUL 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	BRIAN R. STANTON
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PHILLIPS, J.P. et al. Null mutation of copper/zinc superoxide dismutase in Drosophilia confers hypersensitivity to paraquat and reduced longevity. Proceedings of the National Academy of Science, USA. 1989, volume 86, pages 2761-2765, see entire article.	1-16
Y	BENEDETTO, M.T. et al. Isolation and analysis of the mouse genomic sequence encoding $Cu2+-Zn2+$ superoxide dismutase. Gene. 1991, Volume 99, pages 191-195, see entire article.	1-16
Y	CAPECCHI, M.R. The new mouse genetics: Altering the genome by gene targeting. Trends in Genetics, 1989, Volume 5, No. 3, pages 70-76, see entire article.	1-16
Y	DENG H-X et al. Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. Science, 20 August 1993, pages 1047-1051, see entire article.	1-16

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: Aidsline; anabstr; aquasci; biousiness; niosis; biotechds; caba; cancerlit; caplus; ca; ceaba; cen; cin; cjacs; cjelsevier; confsci; cropb; dgene; dissabs; druglaunch; drugnl; drugu; embal; embase; fsta; genbank; healsafe; ifipat; jicst-eplus; jpnews; lifesci; medline; ntis; ocian; phic; phin; promt; scisearch; toxline; toxlit; uspatfull; APS

Search Terms:sod?; superoxide; dismutase?; transgen?; homologous; recombination; embryonic; stem; oxidat?; scott?/au; reaume?/au; hoffman?/au